

Effect of Heat or Chemical Treatment on Leptospiral Antigens

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The use of bacterins is one of the primary methods in the control of leptospirosis in domestic animals, especially cattle, swine, and dogs. Bacterins have been made using chemically treated or heat-inactivated organisms. Many of the initial studies indicated that the bacterins gave complete protection, whereas later reports found that some inactivation methods resulted in bacterins that protect against death but not against kidney infection and renal shedding. This raised the possibility that some of the antigens had been altered. To investigate this possibility, we heat or chemically treated *Leptospira interrogans* serovar *icterohaemorrhagiae* CF-1, *L. interrogans* serovar *canicola* Moulton, *L. interrogans* serovar *grippityphosa* SC4397, *L. interrogans* serovar *hardjo* Hardjoprajitno, and *L. interrogans* serovar *pomona* MLS. Temperatures used to determine alterations in antigens were 50, 56, 80, 100, and 121°C. The chemicals used were formaldehyde, phenol, and thimerosal. Analysis was done using Laemmli gels and Western blots. We found that heating at 50 or 56°C had the smallest effect on antigens, whereas heating at 80 or 100°C caused the appearance of high-molecular-weight bands on Western blots. Heating at 121°C caused almost all the bands to disappear, in both the stained gel and the Western blot. Both phenol and Formalin treatment altered proteins, whereas thimerosal treatment appeared to have little effect compared with heating at 50°C.

Leptospirosis is a zoonotic disease spread by contact with urine from an infected animal or contaminated moist soil. The disease results in abortions and infertility in livestock; in dogs and humans, the disease is primarily a febrile one. Wild rodents serve as the primary reservoir, with some animals remaining infective as renal shedders for life (10). The elimination of these animals is not feasible in the control of leptospirosis; therefore, the most practical approach has been to vaccinate susceptible domestic animals. Since 1950, leptospires for bacterins have been treated with phenol (2), thimerosal (11), Formalin (1, 2, 9), or heat ranging from 50 to 121°C (2, 14). Live avirulent strains of leptospires have been used with moderate success (2, 5, 13) but have not been used routinely because of the possibility of reversion to virulence. Regardless of the bacterin used, all have been successful in preventing death, but efficacy in preventing renal infection and its subsequent renal shedder state has been variable (3). This strongly suggests alterations of some of the immunologically important proteins. To determine what changes occur, we treated various serovars of *Leptospira interrogans* with thimerosal, Formalin, or phenol or temperatures ranging from 50 to 121°C and analyzed the proteins by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12) and Western blot (17).

MATERIALS AND METHODS

Leptospira organisms. *L. interrogans* serovar *canicola* Moulton, *L. interrogans* serovar *icterohaemorrhagiae* CF-1, *L. interrogans* serovar *grippityphosa* SC4397, *L. interrogans* serovar *hardjo* Hardjoprajitno, and *L. interrogans* serovar *pomona* MLS were used. Laboratory-adapted cultures were grown in Tween 80-albumin (T80-Alb; 16)-supplemented medium. When each culture reached the log phase of growth, 5×10^8 organisms, as determined by nephelometer readings (4), were put into each of nine

Microfuge tubes. The cells were centrifuged for 5 min, and the pellets were washed twice in 0.01 M phosphate-buffered saline (PBS) with 10 µg each of CaCl₂ and MgCl₂ per ml (pH 7.2). The pellet in tube 1 was suspended in 100 µl of SDS-PAGE sample buffer–2 µl of bromphenol blue. This was then boiled for 5 min. A 75-µl sample of PBS was added to tube 2 and autoclaved for 10 min at 121°C at 15 lb/in². A 50-µl portion of SDS-PAGE sample buffer and 2 µl of marker dye were added, and the tube was heated at 50°C for 30 min. Cells from tubes 3 through 9 were suspended in 1 ml of PBS. Tube 3 was boiled for 10 min, tube 4 was heated at 80°C for 10 min, tube 5 was heated at 56°C for 30 min, and tube 6 was heated at 50°C for 30 min. After being heated, the cells were spun down, and the pellets were resuspended in 100 µl of SDS-PAGE sample buffer–2 µl of bromphenol blue and heated at 50°C for 30 min. A 10-µl sample of phenol was added to tube 7, 10 µl of Formalin was added to tube 8, and 10 µl of a 1:100 dilution of thimerosal was added to tube 9. The tubes were vortexed and allowed to sit at room temperature for 60 min. The cells were then centrifuged, and the pellets were treated as with tubes 2 through 6.

PAGE. The discontinuous SDS-PAGE method of Laemmli (12) was used with the buffer formulations of Hames (7). Briefly, a 0.375 M Tris hydrochloride (pH 8.8) resolving gel buffer with 10.0% acrylamide and a 0.125 M Tris hydrochloride (pH 6.8)–4% acrylamide stacking gel were used. The electrode buffer was 0.025 M Tris–0.192 M glycine–0.01% SDS (pH 8.3). The lanes were loaded with 25 µl of sample in 0.0625 M Tris hydrochloride–2% SDS–4% 2-mercaptoethanol–10% glycerol (pH 6.8) buffer and run in a cooled vertical gel apparatus at 40 mA per gel until the dye front was approximately 1 cm from the end of the gel. The molecular weight standards (Sigma Chemical Co., St. Louis, Mo.) β-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, and α-lactalbumin were run with each gel. The gels were then either silver stained or immunoblotted.

Silver stain. A combination of the procedures of Poehling and Neuhoﬀ (15) and Guevara et al. (6) was followed for the silver stain, with some modifications. The SDS gel was first

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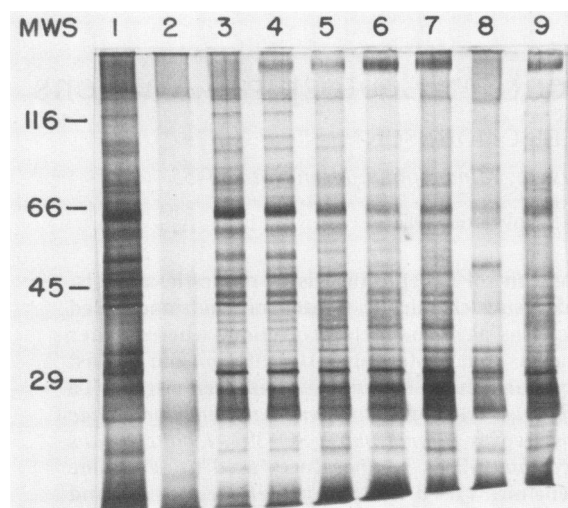


FIG. 1. Silver stain of heat- and chemical-treated *L. interrogans* serovar *hardjo* Hardjoprajtino. For each treatment, 5×10^8 cells were washed and then treated as follows. They were boiled for 5 min in SDS-PAGE sample buffer (lane 1), heated at 121°C in PBS for 10 min (lane 2), heated at 100°C in PBS for 10 min (lane 3), heated at 80°C for 10 min (lane 4), heated at 56°C for 30 min (lane 5), heated at 50°C for 30 min (lane 6), treated with phenol for 60 min (lane 7), treated with Formalin for 60 min (lane 8), and treated with thimerosal for 60 min (lane 9). MWS, Molecular weight standards (10^3).

fixed in 50% methanol–10% acetic acid for 30 min and then in 5% methanol–7% acetic acid for approximately 18 h. The fixing solution was replaced with 10% glutaraldehyde for 30 min. Next, the gel was washed with 20% methanol for a minimum of 90 min with three changes of 30 min each. It was then silver stained for 10 min with a solution of 2 ml of 20% AgNO_3 –10.5 ml of 0.36% NaOH –0.7 ml of NH_4OH –36.8 ml of deionized water. The stain was discarded, and the gel was rinsed with two quick rinses of deionized water and then washed in water for 8 min. After being rinsed, the gel was developed in 100 ml of 0.005% citric acid–50 μl of 37% formaldehyde until the bands were distinct and the background relatively clear. It was then rinsed in several changes of deionized water and photographed on a light box.

Western blot. Electrophoretic transfer of proteins to nitrocellulose was based on the procedure of Towbin et al. (17), with blotting overnight at 100 mA in pH 8.3 Tris glycine buffer–20% methanol. Nonspecific-binding sites were blocked with 3% gelatin–Tris-buffered saline (TBS), with incubation for 45 min. Next was a 45-min incubation with 0.5% Tween 20–TBS (TBS-T20). The antileptospiral antibody was diluted 1:10 in TBS-T20 and incubated for 90 min. The blots were then washed twice, 5 min each, with TBS-T20. The anti-rabbit antibody conjugated to alkaline phosphatase (Cooper Biomedical, Inc., West Chester, Pa.) was diluted according to manufacturer directions in TBS-T20 and incubated for 90 min. This was followed by two 5-min washes with TBS-T20 and one wash with TBS. The substrate reaction mixture consisted of 40 μl of 2 M MgCl_2 , 2 ml of 0.1% Nitro Blue Tetrazolium in pyrogen-free distilled water, 2 mg of 5-bromo-4-chloro-3-indolyl phosphate in 400 μl of dimethylformamide, and 18 ml of 0.15 M sodium barbital buffer. After blotting, the molecular-weight-standard lanes were stained with 0.1% India ink (8).

Antiserum preparation. Log-phase leptospiral organisms

grown in T80-Alb medium were washed three times in sterile 0.01 M PBS (pH 7.2). The final pellet was suspended in sterile physiological saline to a density of 10^8 organisms per ml. Four injections of 0.5, 1.0, 2.0, and 2.0 ml by marginal ear vein were done at weekly intervals. Two weeks after the final inoculation, the rabbits were bled. All antibody titers were determined using the microscopic agglutination test (MAT; 16). The prewashed treated *L. interrogans* serovar *hardjo* antigen preparations were also tested in the MAT using the same rabbit anti-*L. interrogans* serovar *hardjo* antiserum as that used in the Western blots.

RESULTS

The washed cells of all five *L. interrogans* serovars tested were either heat or chemically treated and assayed for changes in their protein profiles. Cells of each serovar were run on a 10.0% Laemmli gel and silver stained. A duplicate gel for each group was also run and transblotted to nitrocellulose sheets (0.45- μm pore size). Because the results with the five serovars were similar, *L. interrogans* serovar *hardjo* was arbitrarily chosen as representative of the group. The silver stain of the prewashed heat- and chemically treated *L. interrogans* serovar *hardjo* cells is shown in Fig. 1. Autoclaving (121°C) distinctly altered most of the proteins. Treatment of cells at 100°C appeared to cause major changes of the protein profile in the 90- to 100-kilodalton (kDa) range. Formalin treatment resulted in the loss of many of the protein bands and alterations in many of the remaining bands. Proteins of the thimerosal-treated cells had a pattern similar to that of the *L. interrogans* serovar *hardjo* cells treated at 50°C. The Western blot (Fig. 2) shows more dramatically the effect of heat on the antigens. Even heating at 56°C eliminated most antibody interactions with high-molecular-mass proteins. Phenol eliminated reactions with proteins above 90 kDa and greatly reduced the reactions

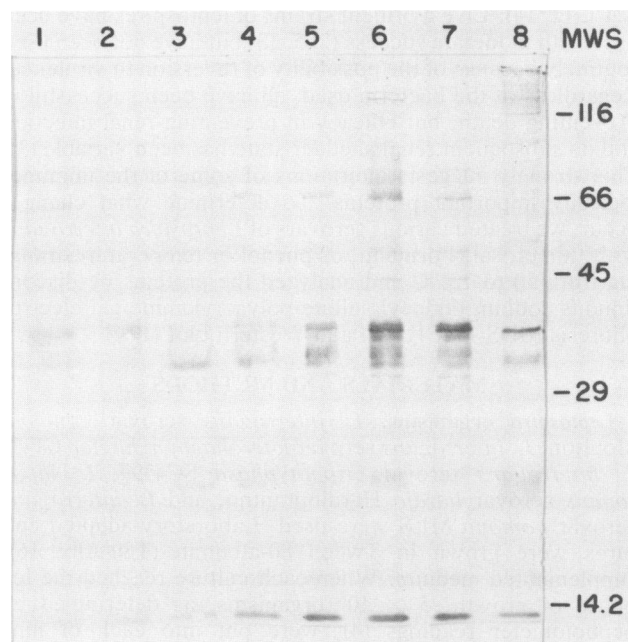


FIG. 2. Western blot of gel shown in Fig. 1. Rabbit anti-*L. interrogans* serovar *hardjo* antiserum and goat anti-rabbit immunoglobulin G (heavy and light chain) conjugated to alkaline phosphatase were used. See legend to Fig. 1 for description of lanes. MWS, Molecular weight standards (10^3).

with the proteins of about 20 kDa. There was an increase in the molecular mass of proteins that reacted with the anti-*L. interrogans* serovar *hardjo* antiserum in the Formalin-treated organisms. The banding patterns of thimerosal- and heat (50°C)-treated cells were very similar (Fig. 3), even when they were treated and then washed.

All sera were tested in the MAT against their native homologous antigens. Heat- and chemically treated *L. interrogans* serovar *hardjo* was also tested in the MAT. When live, unwashed, untreated antigens were used, the homologous *L. interrogans* serovar *hardjo* titer was 1:25,600. Leptospire that had only been washed reacted at a titer of 1:6,400. Heating destroyed some of the reactivity, with decreases in titer corresponding to the increases in temperature. When heated at 100 or 121°C, there was no reactivity at the 1:100 dilution; at 80°C, the titer was 1:800; at 56°C, it was 1:3,200; and at 50°C, it was 1:6,400. There was no reactivity at the 1:100 dilution of the phenol-treated cells, whereas Formalin or thimerosal treatment resulted in a titer of 1:1,600. Agglutination was loose and lacy instead of compact when the leptospire were treated with Formalin or thimerosal or with heat of 80°C.

DISCUSSION

It is known that heat or chemical treatment of leptospire changes their effectiveness as vaccines. An ultrastructure study done in 1965 (18) demonstrated that phenol, Formalin, and thimerosal remove the outer envelope and damage the protoplasmic cylinder. Phenol also results in loose and broken periplasmic flagella. It is known that Formalin-treated antigens have lower sensitivity and greater cross-reactivity in the MAT (16). The effect of other treatments has not been tested in the MAT, and none of these has been previously analyzed using PAGE or Western blotting.

Temperatures in excess of 56°C alter some of the antigens, as observed by SDS-PAGE with silver stain. Treating the leptospire before washing (data not shown) appeared to preserve some of the proteins, because of the presence of bovine serum albumin from the medium, but it also became very difficult to wash the bovine serum albumin off the spirochetes, especially if the preparation was boiled first. Heating at 50°C appeared to be the least disruptive to the integrity of the antigen. Even an increase to 56°C resulted in a loss of some high-molecular-weight molecules.

The effects of the three chemicals on the leptospiral antigens were quite different. With phenol treatment, the high-molecular-mass antigens were altered. Formalin altered the antigens between 29 and 45 kDa, which may account for the lack of sensitivity and specificity in the slide MAT when formalinized cells are used. Thimerosal appeared to have the smallest effect on the antigens, both in their appearance in a silver stain and by Western blot technique. Not washing the cells before treatment seemed to have little effect. This is worth noting, because most bacterins are made with thimerosal-treated, nonwashed serovars grown in T80-Alb medium.

One vaccination study by Borg-Petersen (3) used *L. interrogans* serovar *icterohaemorrhagiae* treated with Formalin or heated, at 56 or 70°C, or washed and then heated at 70°C. All vaccines were effective in preventing death with an immunizing dose of 10^8 organisms. No reference was made regarding the prevention of renal infection. With a dose of 10^7 organisms, effectiveness fell with the heat-treated antigens only. Both the vaccines treated at 70°C were less efficient in preventing death than the vaccine treated at 56°C.

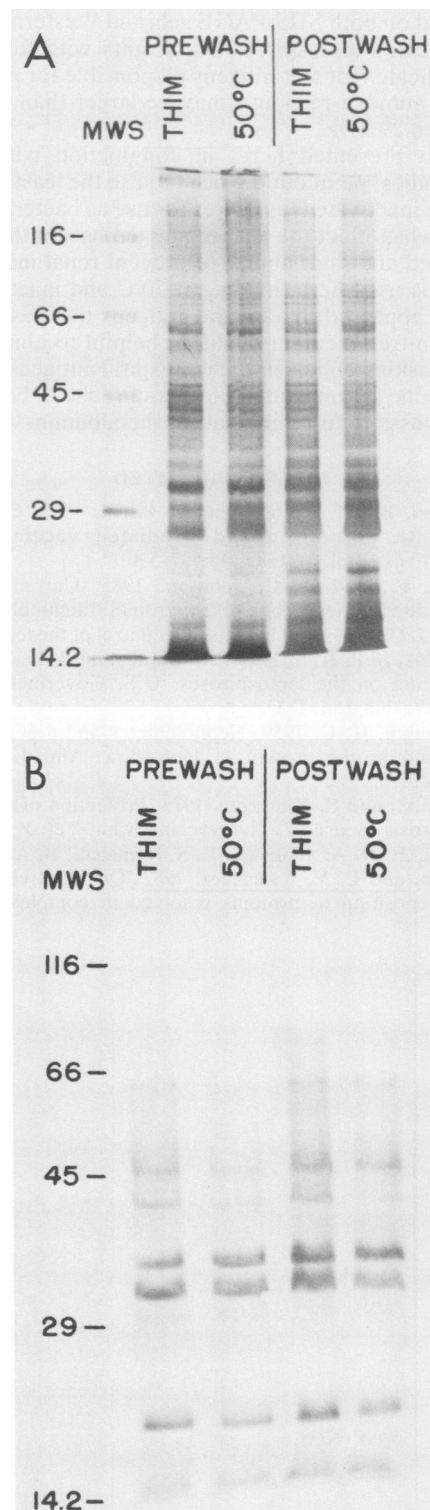


FIG. 3. *L. interrogans* serovar *hardjo* treated with thimerosal or at 50°C, either after or before washing. Panels A and B were run on the same gel. The gel was then halved. The gel in panel A was silver stained, and the gel in panel B was Western blotted. Treatment was as follows: thimerosal for 60 min after washing (lane 1), heating at 50°C for 30 min after washing (lane 2), thimerosal for 60 min before washing (lane 3), and heating at 50°C for 30 min before washing (lane 4). MWS, Molecular weight standards (10^3).

This phenomenon might be partially explained by the results we obtained on both SDS-PAGE gels and Western blots with the heat-treated leptospires. Our results with formalinized antigen indicate that the antigens responsible for most of the protective immune response may be larger than 45 kDa or smaller than 29 kDa.

The data presented here, in conjunction with various vaccine studies, point out the need to use the least disruptive methods to inactivate leptospires for use as bacterins. We do not know what effect the altered antigen has on the antibodies produced and their ability to prevent renal infection. Of the methods used here, heating at 50°C and inactivation by thimerosal appeared to alter the antigens the least. The use of a protein-free medium might be helpful to eliminate any possible masking effects of the leptospiral antigens by adherence of bovine serum albumin to the spirochete, because it is almost impossible to remove all of the albumin.

LITERATURE CITED

1. Babudieri, B., M. Castelli, and F. Pisoni. 1973. Comparative tests with formalized and irradiated vaccines against leptospirosis. *Bull. W.H.O.* **48**:587-590.
2. Bey, R. F., and R. C. Johnson. 1985. Current status of leptospiral vaccines. *Prog. Vet. Microbiol. Immunol.* **2**:175-197.
3. Broom, J. C. 1953. Prophylaxis and control of the leptospiroses, p. 186-201. *In* P. H. Streit, W. S. Stone, and R. H. Yager (ed.), *Symposium on the leptospiroses*. U.S. Government Printing Office, Washington, D.C.
4. Ellinghausen, H. C. 1959. Nephelometry and a nephlo-culture flask used in the growth of leptospira. *Am. J. Vet. Res.* **20**:1072-1076.
5. Fish, N. A., and B. Kingscote. 1973. Protection of gilts against leptospirosis by use of a live vaccine. *Can. Vet. J.* **14**:12-15.
6. Guevara, J., D. A. Johnston, L. S. Ramagali, B. A. Martin, S. Capetillo, and L. V. Rodriguez. 1982. Quantitative aspects of silver deposition in proteins resolved in complex polyacrylamide gels. *Electrophoresis* **3**:197-205.
7. Hames, B. D. 1981. An introduction to polyacrylamide gel electrophoresis, p. 1-91. *In* B. D. Hames and D. Rickwood (ed.), *Gel electrophoresis of proteins: a practical approach*. IRL Press, London.
8. Hancock, K., and V. C. W. Tsang. 1983. India ink staining of protein on nitrocellulose paper. *Anal. Biochem.* **133**:15-62.
9. Hanson, L. E. 1973. Immunologic problems in bovine leptospirosis. *J. Am. Vet. Med. Assoc.* **163**:919-923.
10. Hanson, L. E. 1976. Pathogenesis of leptospirosis, p. 295-316. *In* R. C. Johnson (ed.), *Biology of the parasitic spirochetes*. Academic Press, Inc., New York.
11. Hodges, R. J. 1977. *Leptospira interrogans* serotype pomona infection in pigs: prevention of leptospirosis by immunization before exposure to a natural infection. *N.Z. Vet. J.* **25**:33-35.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
13. Morsi, H. M., and G. P. Shibley. 1973. Cellular and whole culture bivalent leptospiral bacterins: safety and potency in guinea pigs and hamsters. *Am. J. Vet. Res.* **34**:15-16.
14. Painter, G. M., and H. C. Ellinghausen. 1976. Immunizing potency of *Leptospira interrogans* serotype canicola after heat inactivation at different temperatures. *J. Med. Microbiol.* **9**:487-492.
15. Poehling, H. M., and V. Neuhoff. 1981. Visualization of proteins with a silver "stain": a critical analysis. *Electrophoresis* **2**:141-147.
16. Sulzer, C. R., and W. L. Jones. 1980. Leptospirosis: methods in laboratory diagnosis. U.S. Department of Health, Education, and Welfare publication no. (CDC) 76-8275, p. 12-15. Center for Disease Control, Atlanta.
17. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4353.
18. White, F. H., and C. F. Simpson. 1965. The effect of formalin and other inactivators on the ultrastructure of leptospires. *J. Infect. Dis.* **115**:123-130.